原 著

Characterization of a YAC Clone Exhibiting Unique Behavior of Abnormal Chromosomal Distribution

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＜Abstract＞

Another unique clone was found in the genomic library that was constructed specifically for human chromosome 21 by us previously. The YAC (yeast artificial chromosome) clone exhibited unusual chromosomal distribution different from our previous report. Though it showed no extra band specific to an artificial chromosome by means of karyotype analysis with CHEF, human specific short-repetitive-sequence, *Alu* hybridized to two bands (250kb and 125kb). It was presumed that different clones are contaminated in that clone, since a single yeast cell usually does not contain several artificial chromosomes. Thus, as we reported previously, individual colonies were isolated from the original clone and CHEF analysis was performed for them through six generations. As a result, it was shown that seven different karyotypes, including the original clone itself, are generated from the original one. The original clone is considered to be lacking functioning of regulatory factors for normal chromosomal distribution. There is a possibility that novel regulatory factors could be identified from further studies of these unique karyotypes. We report in this article the analytical result on the abnormal behavior in chromosomal distribution of this newly found clone.

Keywords: YAC, Chromosomal distribution

INTRODUCTION

Saccharomyces cerevisiae is a powerful tool as a model system to define the chromosomal instability phenotype [1]. Chromosomal instability is largely caused by disruption of regulatory system for DNA recombination event on meiosis. Meiosis involves the formation of homolog pairs of DNA, and then the chromosomes are connected by a specific structure: the synaptonemal complex. The interaction between homologs includes recombination at the DNA level, which is initially introduced by double-stranded break (DSB) of DNA. An interesting fact is that the meiotic recombination event called as crossover is strictly regulated to occur on more than one place of a single chromosome (obligate crossover). And also the event occurs on only few places of a single chromosome [2] (crossover interference). Defects in the genes related to meiotic recombination are lethal for most of the organisms. For human, defects in these essential genes cause critical disorder in chromosome number and its structure, but in most cases embryos fall in death with several exception such as Down syndrome (trisomy or partial deletion of chromosome 21), Turner syndrome (trisomy of chromosome X), Klinefelter syndrome (monosomy of chromosome Y) and so on [3].

We have been working to obtain a unique yeast mutant that shows chromosomal instability phenotype to get a clue to identify one of the novel key factors controlling the chromosomal replication. We found a very unique clone among the YAC genomic library that was constructed for human chromosome 21 specific genomic DNA [4]. The YAC clone generated four different derivatives by single-colony-isolation, showing four different chromosomal karyotypes including the original clone [5]. The interesting feature

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of this clone was that the manner of the derivative generation seemed to follow a rule. A clone expressing such a unique chromosomal behavior has not ever been reported before. During the process of characterizing YAC clones, another unique YAC clone showing abnormal chromosomal distribution was found. The analytical results on the newly found unique clone are reported in this article.

MATERIALS AND METHODS

Yeast strains, plasmids, and chemicals.

Saccharomyces cerevisiae strain AB1380 (Mat-a, ade 2-1, can 1-100, lys 2-1, trp 1, ura 3, his 5[psi+]), pYAC 55, and the HY-1 YAC clone (125-kb DNA fragment from EB virus transformed human peripheral lymphocytes [6]. Chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA except ones indicated below.

YAC selection

Single colony that possesses YAC was isolated on the double selection medium (Ura^- and Trp^-), SD agar plate. The composition of SD medium is 0.17% yeast nitrogen base without amino acids, 0.5% ammonium persulfate, 0.056% amino acid mix without uracil and tryptophan [7], 2% glucose and 2% agar (pH5.8).

Contour-clamped homogenous electric field (CHEF) gel electrophoresis.

CHEF gel electrophoresis was performed as described elsewhere [8], using a CHEF-DR III system (Nippon Bio-Rad Laboratories, Fukuoka, Japan). The 1% agarose gels in 0.5× TBE (45mM Tris-borate, 45mM boric acid, 1mM pH8.0EDTA) were prepared by pouring 100 ml agarose (Agarose NA, Pharmacia AB, Uppsala, Sweden) into a 12×12㎠ frame. Electrophoresis was carried out in 0.5× TBE as running buffer at a constant temperature at 14℃, which was maintained by re-circulation of the buffer through a heat exchanger, at a constant voltage of 170V for 20 hrs. The stepped switching interval was 60sec for the first 12hr and 90sec for the next 8hrs.

Preparation of agarose embedded yeast DNA

A single colony on YPD (1% yeast extract, 2% peptone, 2% glucose, pH5.8) agar plate was inoculated into 2 ml YPD broth. After grown for 20hrs to stationary growth phase, the cells were collected by centrifugation at 5,000× g, 5min, at 25℃. The supernatant was decanted and the cells were re-suspended in 1ml SE (75mM NaCl, 25mM pH7.4EDTA). Cells were collected again by centrifugation at 5,000× g, 5min, at 25℃. 50μl of SE and 150μl of agarose suspension buffer (ASB) which was equilibrated to 55℃ in a water bath were added. ASB was freshly prepared in each experiment. 200μl of ASB was mixed with following four solutions, 500μl of 3.5% low melt agarose (BRL, Gaithersburg, MD, USA) which was equilibrated to 55℃ in a water bath after autoclaved in SE for 1min at 121℃, 20μl of 1M DTT (filter-sterilized after dissolved in water), 10μl of 1mg/ml Zymolyase (Kirin 100T, Kirin Brewery Co. Ltd, Tokyo, Japan) suspended in sterile water, and 470μl of SE. After immediately combining the cell mixture, transfer the mixture plug mold and solidify for 8min at −20℃. Each solidified agarose plug was pushed into a well of 20 well micro plate and 80μl of 1M DTT (filter-sterilized after dissolved in water), 40μl of 1mg/ml Zymolyase (Kirin 100T, Kirin Brewery Co. Ltd, Tokyo, Japan) suspended in sterile water, 1880μl of SE were added into each well. After incubated for 1hr at 37℃, each agarose plug was washed with ES (0.5M pH9.5EDTA, 1% sodium sarcosinate) twice for 10min. 1ml ES and 50μl of proteinase K (Merk, Darmstadt, FRG) solution (10mg/ml in 50% glycerol) was added to each well and incubated at 55℃ for 20 hrs. After proteinase K treatment, each agarose plugs were washed with 1× TE (20mM Tris-HCl, 1mM EDTA, pH7.4) twice for 20min and stored at 4℃ until use.

Southern blot analysis

CHEF gels were treated with 0.25N HCl for 15 min, then with a solution of 0.4N NaOH and 0.6M NaCl for 15min, and finally with a solution of 1.5M NaCl and 0.5M Tris-HCl (pH 7.5), before transfer to nylon membranes (Pall, Glen Cove, NY, USA) as described elsewhere [9]. Hybridization was carried out in a solution of 1M NaCl, 10% dextran sulfate and 1.0% sodium dodecyl sulfate with

100 μg/ml yeast tRNA as carrier and approximately 10^6 cpm/ml of ^{32}P-labeled probe. The probes used were a 280-bp *Bam*HI fragment of BLUR-8 (*Alu* probe) [10], 2670-bp *Pvu*II / *Bam*HI digest of pBR322 (left arm probe) and 1692-bp *Bam*HI / *Pvu*II digest of pBR322 (right arm probe), radiolabeled with [^{32}P] dCTP with an oligopriming labeling kit [11] (Pharmacia-LKB). Membranes were washed twice with 2× SSC at room temperature for 10min, then once with 2× SSC, 1% SDS at 65℃ once for 30 min, and finally once with 0.1× SSC at room temperature once for 30min. Autoradiography was performed at −80℃ for a day. Filter was served for re-hybridization after labeled probe was completely washed off with the treatment of boiling for 30min in 0.1× SSC.

RESULTS AND DISCUSSION

The YAC (yeast artificial chromosome) cloning system is a distinguished vector system utilizing the artificial chromosome which allows cloning of large fragment of exogenous DNA that is several hundred kilobases in length [6]. A schematic view of the cloning system is shown in Fig.1. The vector incorporates all necessary functions into a single plasmid that can replicate in *Eschericia coli*. This plasmid is called as a "yeast artificial chromosome" (YAC) vector. Cleavage at *Bam*HI sites adjacent to the TEL sequences produces termini that heal into functional telomeres *in vivo*. And ARS (ARS1) and a centromere (CEN4) enable the inserted DNA fragment to replicate as a normal yeast chromosome.

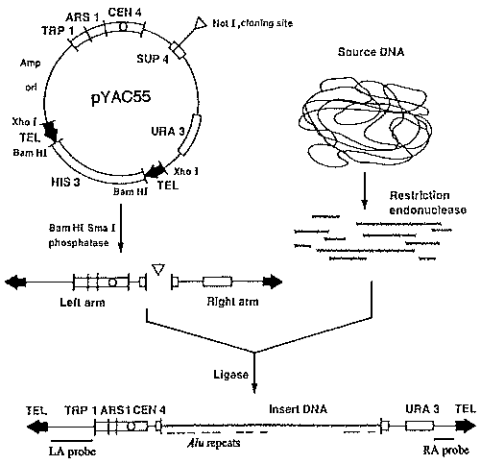

Fig. 1. Yeast artificial chromosome (YAC) cloning system. Since the YAC clone 6H1 was selected with *Alu* probe from the pool of genomic library that was constructed with a mixed Chinese hamster and human chromosome 21 genomic DNA [2], *Alu* should exist in the cloned insert fragment even if the genomic nucleotide sequencing has not yet been performed. Thus, the position of *Alu* sequence is drawn on the inserted fragment at random. "LA" and "RA" indicate the probe position on the left arm and right arm of the vector, respectively.

Among 9930 *Alu* (human specific short-repetitive-sequence) positive clones that were detected by dot hybridization technique within the pool of YAC genomic libraries [4], 7 clones showed abnormal features in the Southern blot of the CHEF gel that was probed with *Alu*. As shown in Fig.2, the YAC clone 13F3 has lost the 280kb normal chromosome, and both the 130kb and 200kb bands were newly built chromosome. *Alu* probe did not hybridize any chromosome, but the right vector arm hybridized to the 125kb band and the left vector arm hybridized to the 200kb band. This result possibly means that 280kb chromosome has split out into two smaller chromosomes by some reason. The YAC clone 14E9 has a newly built 500kb chromosome. *Alu* probe and the right vector arm hybridized to the chromosome, but the left vector arm did not hybridize to it. In this case, the left vector arm must be integrated into the normal 970 kb chromosome. This phenomenon would be caused by the chromosomal recombination between the 970 kb chromosome and the 500 kb artificial chromosome that has a part of human genome. Each of the YAC clone 22E10 and the 23D12 has a newly built 470kb chromosome. But *Alu* probe did not hybridize to them. In these clones, the right vector arms hybridized to both the newly built chromosomes and the 970kb chromosome, and the left vector arm hybridized to the 970kb chromosome. In these two clones, the vector arms must have integrated into the yeast's conventional chromosomes forming a new chromosome after a chromosomal recombination event. The YAC clone 3G6 has no trace of an artificial chromosome in the CHEF gel, but *Alu* and both the vector right arm left arms hybridized to the 360kb chromosome. If both of the 360 kb artificial chromosome and the normal 360 kb chromosome of yeast exist in this clone, the 360 kb band should express doubled intensity in the strained CHEF gel,

but it can not be observed. Therefore, most possible explanation for this clone is that a human genomic DNA and the vector arms are integrated into the 360 kb yeast's chromosome. The abnormal karyotypes of these 5 YAC clones did not change after the treatment of single-colony-isolation.

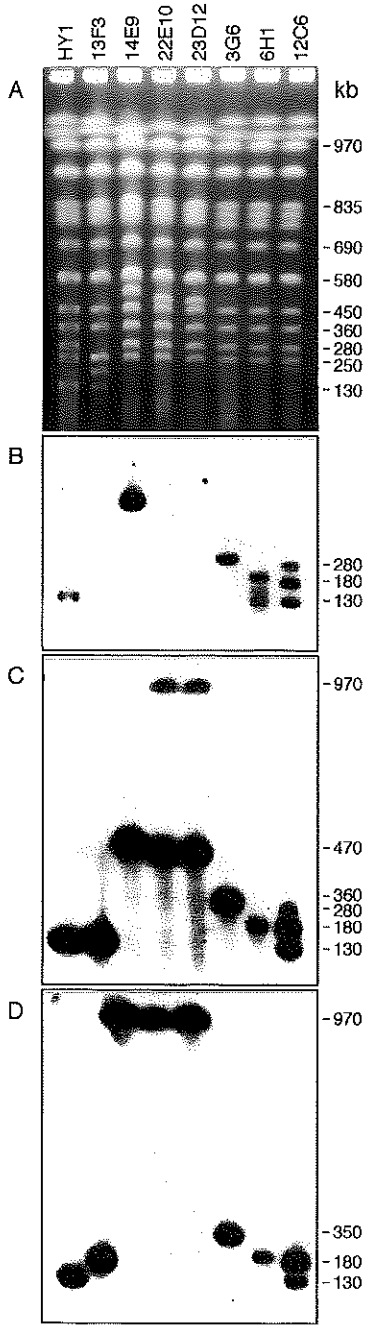

Fig. 2. CHEF analysis of the abnormal YAC clones. A: a gel stained with ethidium bromide. B, C and D: Southern blots with *Alu* probe, LA probe and RA probe, respectively. The HY-1 is the marker strain of YAC clone that harbors an artificial chromosome of 125kb size derived from human genome.

The YAC clone 12C6 was analyzed minutely in our previous study [5]. The YAC clone 6H1 is a newly found clone that shows similar features to the YAC clone 12C6. Though it showed no extra band specific to an artificial chromosome in karyotype analysis with CHEF as shown in Fig.2A, *Alu* hybridized to three bands (250kb, 200kb and 125kb), but two of which (200kb and 125kb) are invisible in the CHEF karyotype. In this clone, both of the right and left arms hybridized to 250 kb chromosome. In order to examine whether it was due to contamination of other yeasts or due to a regulatory pattern in the unstable chromosomal distribution on each single-colony-isolation treatment, we repeated single-colony-isolation together with the CHEF karyotype analysis through six generations as described in our previous report [5] As the result, we confirmed that there are 7 karyotypes including the original clone generated from the YAC clone 6H1 (they are named as the Original and Type1-6).

Fig.3 shows the result of CHEF analysis and southern hybridization for the 7 karyotypes obtained from the YAC clone 6 H1 after single-colony-isolation. The HY-1 marker strain has an artificial chromosome of 125 kb size derived from human genome and three probes (*Alu*, LA and RA) hybridized to the 125kb band, suggesting that this clone is a stable and typical YAC clone. The Original showed two extra bands of 250kb and 125kb on the stained CHEF gel as shown in Fig.3A. In Fig.2A, the original clone had another extra 200kb band but it disappeared after repeated single-colony-isolation. It is unclear whether the initially recognized 200kb band is caused by contamination of any derivatives of the 6H1 clone or the band has disappeared by some unknown reason after repeated single-colony-isolation. If the extra 250kb band is overlapped with the yeast host 250kb chromosome, the intensity of the 250kb band should be stronger than the other chromosomes in Fig.3A, but such a sign can't be seen in the result. Therefore it is postulated that human genomic DNA and the vector arms are integrated into the yeast's host 250kb chromosome.

Type 1, Type 2 and Type 3 seem to be normal

YAC clones since *Alu* and both right and left vector arms hybridized to each of the single chromosome of 240kb, 130kb and 115kb, respectively. Each of Type 4, Type 5 and Type 6 had a single artificial chromosome of 135kb, 180kb and 195kb, respectively in the stained CHEF gel. But for Type 4, *Alu* hybridized to the 135kb and 200kb bands. The 200kb band of Type 4 is not seen in the stained CHEF gel (Fig.3A) and the left vector arm hybridized to the band but the right vector arm did not. As for Type 5, *Alu* and both the right and left vector arms hybridized to the 180kb chromosome. But the left vector arm also hybridized to the 200kb band that is invisible in the strained CHEF gel. As for Type 6, *Alu* and the left vector arm hybridized to both 195kb and 250kb chromosomes, but the right vector arm hybridized to none.

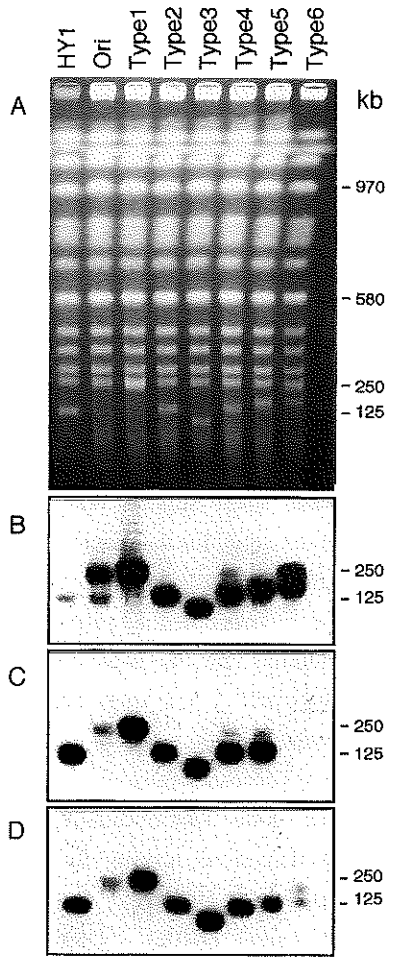

Fig. 3.CHEF analysis of the 7 derivatives from the YAC original clone 6H1. A: a gel stained with ethidium bromide. B, C and D: Southern blots with *Alu* probe, LA probe and RA probe, respectively. The HY-1 is the marker strain. Ori means the original YAC clone 6H1. Type 1-6 is the derivatives from the original clone.

Each of the six derivatives (Type1-6) is served for single-colony-isolation to see what types of karyotype would be produced. Type 1, Type 3 and Type 4 did not produce any derivatives as shown in Fig.4. This suggests that these three derivatives show stable karyotypes. But Type 2, Type 5 and Type 6 produced several karyotypes as shown in Fig.5. Type 2 produced karyotypes of the Original, Type 2, Type 5 and Type 6. Type 5 produced karyotypes of Type 2, Type 4, Type 5 and Type 6. Type 6 produced karyotypes of the Original, Type 1 and Type 6.

These six karyotypes are generated from a single original YAC clone. The question is the reasons why chromosomal distributions of the Original, Type 2, Type 5 and Type 6 are unstable while those of Type 1, Type 3 and Type 4 are stable. The events of homologous DNA recombination or DNA deletion are caused by co-operative action of many factors related to cell division. For example, they are the factors for DNA replication and DNA repair, cyclins, condensins, cohisins, adherins, separins, securins, and so on. The known functions of these factors must be only a part of the whole event process. The Original, Type 2, Type 5 and Type 6 are lacking the function of normal chromosome distribution at the somatic cell division. Interestingly, the defect is recovered in Type 1, Type 3 and Type 4. We speculate that a recombination hot spot existing in the inserted human DNA is deleted in Type 1, Type 3 and Type 4, resulting in stable chromosomal condition. In this speculation, it is possible that the recombination hot spot of the inserted human DNA was associated with the specific DNA sequence of the yeast host genome. The gene containing the recombination hot spot could be identified by means of mRNA subtraction between stable cells and unstable cells, or by means of the complementation of the unstable cells.

There are several recombination hot spots reported. One of the hot spots is LCRs [12] (low-copy repeats). LCRs are usually of 10-400 kb in size and exhibit 95-97 % similarity in human genome. LCRs

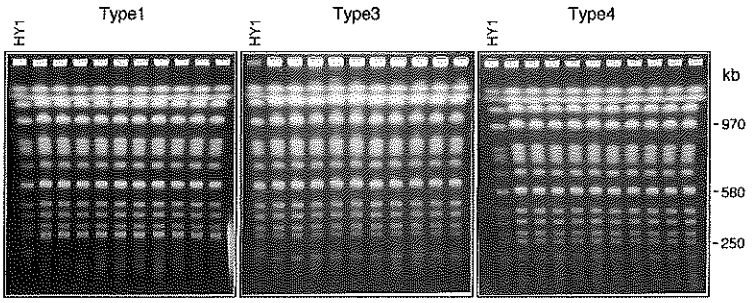

Fig. 4. CHEF analysis of the randomly selected 10 colonies after single-colony-isolation of Type1, Type 3 and Type 4 karyotypes.

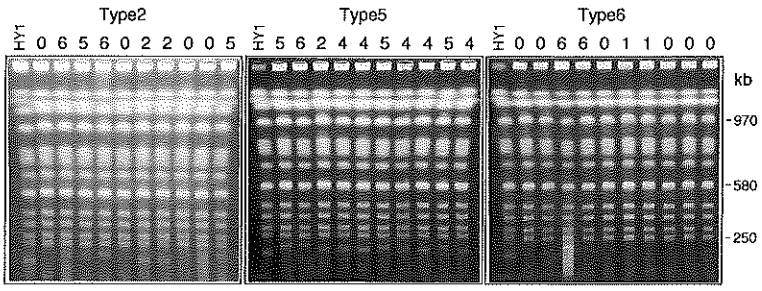

Fig. 5. CHEF analysis of the randomly selected 10 colonies after single colony isolation of Type2, Type 5 and Type 6 karyotypes. Numerous number on the top of each lane indicates the derivative type number. 0 indicates the original YAC clone 6H1.

constitute about 5% of the human genome. LCRs seem to play a major role in both primate karyotype evolution and human tumorgenesis. *Alu* repeat clusters also mediate chromosomal rearrangements [13]. Other repetitive sequences like SINEs (short interspersed elements) and LINEs (long interspersed elements) might play important role. The other hot spot is highly GC rich region [14]. Another hot spot is the MHC (major histocompatibility complex) gene locus that has a single-nucleotide polymorphism [15]. Further analysis on this YAC clone might lead to a discovery of a novel hot spot for DNA recombination.

It is reported that the deletion of RAD52 enhances the chromosomal translocations in budding yeast [16]. RAD52 is a RecA like protein and plays an essential role on DNA replication, DNA repair, and DNA recombination. The RAD52 gene is highly conserved in mammalian genome including human. It might be possible that the function of RAD52 or the function of some other proteins working together with RAD52 (such as MRE11, NBS1, *etc*) is blocked in the Original, Type 2, Type 5 and Type 6 karyotypes for some reason. And the block is possibly unlocked in Type 1, Type 3 and Type 4 karyotypes.

In a somatic chromosome division, homologous recombination contributes to repair DNA damage of DSB during the cell division cycle between late S-phase and G2-phase [17]. Non-homologous end-joining (NHEJ) is another rescue system from DNA damage caused on between G1-phase and early S-phase. These facts suggest that homologous recombination is essential for both meiosis and mitosis. One of the hallmarks of tumor cells is their highly rearranged karyotypes with respect to both chromosome number and the structural integrity of each homologous pair. And there are also several human diseases caused by chromosomal rearrangements. These chromosomal rearrangements are consequence of the loss of fidelity in repairing DSBs. These dangerous lesions are corrected by two primary pathways of NHEJ and homologous recombinational repair (HRR). Disruption of the genes such as RAD51, MRE11, RAD50 and NBS1 that are related to homologous recombination is lethal in mammalian [18], but not in budding yeast. Thus it is interesting to see how these factors correlated to the karyotypes found in this study.

CONCLUSION

We have been aiming to obtain useful mutants to identify novel factors associated with homologous recombination event. In previous [5] and this report, as candidates for our targeting mutants, we showed the behavior of two unique clones exibiting unstable chromosomal distribution. The clones were identified from a human chromosome 21 equivalent library [4] which was constructed from a line of human-rodent somatic hybrid cells with *Alu* selection by conventional hybridization techniques [19]. Difficulty in the yeast genetic approach to find out the causative genes for highly unstable and patterned abnormal chromosome distribution, is the presence of exogenous human DNA in these clones. It disturbs most trials of the yeast's conventional genetic techniques to identify targeting genes. We will continue to try to find out novel key factors controlling stable chromosome distribution using these clones and at the same time we will make efforts to obtain similar mutants from intact yeast cell having no exogenous DNA.

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REFERENCES

1) Putman, C.D., Pennaneach, V. and Kolodner R. D.: *Saccharomyces cerevisiae* as a model system to define the chromosomal instability phenotype. *Mol Cell Biol.* 25:7226-7238, 2005.
2) Zickler, D. and Kleckner, N.: Meiotic Chromosomes: Integrating structure and function. *Annu. Rev. Genet.* 33:603-754, 1999.
3) Watanabe,Y.: The connection of DNA replication and chromosome segregation in meiosis. *Cell Technol.* 22:262-266, 2003.
4) Kai, T. *et al.*: Rapid method for construction of yeast artificial chromosome human DNA libraries involving the trapping of cells in agarose films. *FEBS Lett.* 275:77-82, 1990.
5) Kai, T. and Tatsuguchi, K.: Unique behavior of abnormal chromosomal distribution exhibited by a human Ch.21 YAC. *Seinan-Jo Gakuin Univ. Bulletin* 9:95-103, 2005.
6) Burk, D.T., Carle, G.F. and Olson, M.V.: Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors. *Science* 236:806-812, 1987.
7) Kai, T., Tashiro, H. and Soeda, E.: YAC vector: Cloning of high molecular DNA with yeast artificial chromosome. *Exp. Med.* 8:85-91, 1990.
8) Chu, G., Voiirath, D. and Davis, R.W.: Separation of large DNA molecules by contour-clamped homogenous electric field. *Science* 234:1582-1585, 1986.
9) Carle, G.F., Frank, M. and Olson, M.V.: Electrophoretic separations of large DNA molecules by periodic inversion of the electric field. *Science* 232:65-68, 1986.
10) Deininger, P.L. *et al.*: Base sequence studies of 300 nucleotide renatured reported human DNA clones. *J.Mol.Biol.* 151:17-33, 1981.
11) Feinberg, A.P. and Vogelstein, B.: A technique for radolabeling DNA restriction fragments to high soecific activity. *Anal. Biochem.* 132:6-13, 1983.
12) Stankiewicz, P. L. JR.: Molecular-evolutionary mechanisms for genomic disorders. *Curr. Opin. Genet. Dev.* 12:312-319, 2002.
13) Kolomietz, E. *et al.*: The role of *Alu* repeat clusters as mediators of recurrent chromosomal aberrations in tumors. *Genes Chromosomes Cancer* 35:97-112, 2002.
14) Kong, A. *et al.*: A high-resolution recombination map of the human genome. *Nat. Genet.* 31:241-247, 2002.
15) Jeffreys, A.J. and Neumann, R.: Reciprocal crossover asymmetry and meiotic drive in a human recombination hot spot. *Nat. Genet.* 31:267-271, 2002.
16) Tennyson, R.B. *et al.*: A novel selection system for chromosome translocations in *Saccharomyces cerevisiae*. *Genetics* 160:1363-1373, 2002.
17) Nasmyth, K.: Dissemination the genome: Joining, resolving, and separation sister chromatids during mitosis and meiosis. *Annu. Rev. Genet.* 35:673-745, 2001.
18) Thompson, L.H. and Schild, D.: Recombinational DNA repair and human disease. *Mutat. Res.* 509: 49-78, 2002.
19) Brownstein, B.H. *et al.*: Isolation of a single-copy human gene from a library of yeast artificial chromosome clones. *Science* 224:1348-1351, 1989.

染色体異常分配挙動を示すYACクローンの特性解析

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＜要　旨＞

我々が先に構築したヒト21番染色体特異的なゲノムライブラリーの中から、ユニークなクローンが新たに発見された。このYAC（酵母人工染色体）クローンは前報とは違った異常な染色体挙動を示した。このクローンはCHEFによる核型分析では人工染色体と思われる特別なバンドは示さなかったが、ヒト特有の短鎖反復配列である*Alu*が二つのバンド（200 kbと125 kb）にハイブリダイズした。一般にはひとつの酵母細胞に複数の人工染色体が存在することは無いので、クローンのコンタミネーションが疑われた。そこで前報と同じようにオリジナルクローンから単一コロニーを6世代に渡って分離し、その各世代でCHEF解析を実施した。その結果、オリジナルクローンから、それ自身も含めると7種のカリオタイプが派生することが分かった。このオリジナルクローンでは、染色体分配を正常に制御する因子が機能しないために異常分配を引き起こしているものと考えられる。これらのカリオタイプを用いて染色体分配に関わる新たな因子の同定が期待される。本稿では、この新たに分離されたクローンの異常な染色体分配挙動の解析結果を報告する。

キーワード：ＹＡＣ、染色体分配